Characterization of Human Endothelial Cell Urokinase-Type Plasminogen Activator Receptor Protein and Messenger RNA

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Human umbilical vein endothelial cells in culture (HUVEC) express receptors for urokinase-type plasminogen activator (u-PA). The immunological nature of this u-PA receptor and its relationship to u-PA receptors expressed by other cell types is unknown. Cross-linking active site-blocked u-PA to HUVEC lead to an increase in its apparent molecular mass by approximately 40 Kd. The predominant u-PA binding protein isolated from whole cell detergent extracts migrated with a molecular mass of approximately 36 Kd using affinity chromatography. In contrast, when only cell surface proteins were radiolabeled before extraction, the predominant labeled u-PA binding protein isolated migrated with a molecular mass of approximately 46 Kd. Several pieces of evidence suggested that the difference in molecular mass between these two u-PA binding proteins resulted from glycosylation of a single receptor protein. First, a polyclonal antibody against u-PA receptor isolated from phorbol myristate acetate (PMA) stimulated U-937 cells reacted with both the 36- and 46-Kd proteins on Western blotting. Second, the size of the unmodified receptor was estimated by amplifying a full-length cDNA for u-PA receptor from an endothelial cell cDNA library using the polymerase chain reaction (PCR) and oligonucleotide primers corresponding to the DNA sequence of the receptor cloned from transformed human fibroblasts (Roldan et al, EMBO J 9:467, 1990). The size of the cDNA (~1,054 base pairs, bp) and the presence of a single 1.4-kilobase (Kb) mRNA transcript on Northern blot analysis predict an unglycosylated receptor protein of approximately 35 Kd. Third, synthesis of ^3S-labeled 46-Kd cell surface receptor protein was inhibited when the cells were grown in the presence of tunicamycin, while the synthesis of the 36-Kd species was unaffected. Moreover, the apparent molecular mass of purified surface-labeled receptor (~46 Kd) was reduced by N-glycanase. These studies suggest that the u-PA receptor on the surface of HUVEC is a glycoprotein derived from a protein of approximately 36 Kd which is similar immunologically to u-PA receptors on other cell types.

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PLASMINOGEN activators (PAs) are serine proteases of restricted substrate specificity that have been implicated in a variety of biologic processes, including fibrinolysis, placental implantation, and migration of normal and malignant cells. The proteolytic activity of these enzymes is tightly controlled by a series of fast-acting inhibitors of the activators themselves and inhibitors of plasmin. Because the inhibitors are present in higher concentration than the PAs in plasma and in those sites in which they have been measured, such as subcellular matrix, it is uncertain how PA activity is expressed and localized before the enzymes are inactivated.

PAs may be protected from inactivation through binding to specific receptors on cell surfaces. We and other investigators have reported that human endothelial cells in culture (HUVEC) express high-affinity binding sites for urokinase-type PA (u-PA), and studies from our laboratory and others suggest that cell-bound single-chain u-PA (scu-PA) expresses PA activity when cleaved by plasmin, although functionally active PA inhibitors (PAs) are present in the cell matrix. The receptor for u-PA expressed by endothelial cells has not been isolated previously. Hajjar and Hamel observed that u-PA binds to a protein of approximately 48 Kd in HUVEC extracts identified using ligand blotting. The relationship between this protein and u-PA receptors isolated from other cell types has not been established.

Recently, Roldan et al isolated a 1.4-kilobase (kb) cDNA clone from a transformed fibroblast cDNA library which encodes for a u-PA receptor expressed by phorbol myristate acetate (PMA)-stimulated U-937 cells. The sequence includes a 46-nucleotide 5' untranslated region, a 1,005-nucleotide open reading frame coding region, and a 349-nucleotide 3' untranslated region which includes the polyadenylation sequence. Based on the length and sequence of the cDNA, the mature unglycosylated protein (after cleavage of a putative signal peptide) would be expected to have a molecular mass of 34.6 Kd. However, the u-PA binding proteins isolated from a variety of tumor cell lines and identified in HUVEC by ligand blotting are heterogeneous in apparent molecular masses, and are larger than would be predicted based on this sequence. This increase in the actual observed mass has been attributed to differences in glycosylation. Moreover, the reported affinity of u-PA for endothelial cells is somewhat lower than that reported for U-937 and HeLa cells. Thus, the relationship between u-PA receptors expressed on tumor cell lines and the receptor expressed by human endothelial cells is uncertain. Therefore,
we wished to isolate the u-PA receptor protein expressed by endothelial cells and to determine its relationship to u-PA receptors expressed by other cell types.

**MATERIALS AND METHODS**

**PAs**

Natural scu-PA (55 Kd) was purified from the conditioned medium of HEK cells using zine chelation-Sepharose followed by SP-Sephadex C-50, Sephadex G-100, and benzamidine-Sepharose chromatography as described previously.24 Purified two-chain high-molecular-weight (mol wt) urokinase (tcu-PA) was purchased from Behring Diagnostics. Purified amino-terminal fragment (ATF, 19 Kd) containing amino acids 1 through 143 was prepared by allowing limited proteolysis of scu-PA to occur spontaneously after the SP-Sephadex purification step. ATF was separated from the low mol wt u-PA fragment using benzamidine-Sepharose in a buffer containing 0.1 mol/L 3-[N-Morpholino]propanesulfonic acid (Sigma Chemical, St. Louis, MO), 0.4 mol/L NaCl, and 0.01% Tween 80 (pH 8.0). Recombinant tissue-type PA (rt-PA) was a gift from Genentech (South San Francisco, CA). To block the catalytic site of tcu-PA (DIP-tcu-PA), the protein was incubated with DFP for 30 minutes at 22°C twice (final concentration 20 mmol/L), which resulted in loss of greater than 95% of its amidolytic activity. Protein concentrations were determined using the BioRad protein assay kit. Protein bound to u-PA-Sepharose gel or control Sepharose gel was eluted with 0.1 mol/L NaCl or 0.1 mol/L Tris-HCl (pH 8.5) with a typical coupling efficiency of >95% as assessed by OD_{280}. After the u-PA-coupled gels had been washed extensively, labeled or unlabeled extracted proteins were incubated with the gel for 16 hours at 4°C. Control experiments were performed in parallel using uncoupled Sepharose to determine the amount of nonspecific trapping by the Sepharose beads. When radiolabeled proteins were analyzed, the gel was washed extensively until the eluted radioactivity reached a stable background. Residual proteins bound to the u-PA-Sepharose gel or control Sepharose gel were eluted with either 50 mmol/L glycine buffer (pH 3.0) or 50 mmol/L citrate buffer (pH 3.0) in 0.1 mol/L NaCl and neutralized immediately with one-fifth vol HEPES buffer (pH 9). The eluted proteins were concentrated using Amicon YM-10 concentrating filters (W.R. Grace, Danvers, MA) before further analysis. In some experiments, u-PA binding proteins were first isolated using u-PA-Sepharose. The proteins were then labeled with Na\textsuperscript{125}I and iodogen beads and analyzed using SDS-PAGE and autoradiography. Gels used to analyze 	extsuperscript{125}I-labeled proteins were treated with Autofluor (National Diagnostics, Manville, NJ) before autoradiography.

**Isolation of Receptor Protein**

HUVEC in stationary culture for 48 to 72 hours after the final passage were washed once in cold PBS containing 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). The cells were incubated briefly with 5 mmol/L EDTA and then gently scraped. The cell suspension was pelleted and resuspended to a final concentration of 1 x 10\textsuperscript{7} cells/mL in extracting buffer containing 0.5% CHAPS detergent (Pierce), 1 mmol/L PMSF, 10 mmol/L EDTA, 10 μg/mL aprotinin, and 0.1 mol/L Tris-HCl (pH 8.1) for 1 hour at 4°C. The mixture was then centrifuged at 100,000g for 1 hour, and the supernatant was frozen at -70°C until use.

Affinity columns were prepared using Reacti-Gel 6x (Pierce) to which scu-PA was linked. Ten milligrams of protein was incubated with 5 mL of gel in 0.1 mol/L borate buffer containing 0.01% Tween 80 and 1 μg/mL aprotinin, pH 8.5 with a typical coupling efficiency of >95% as assessed by OD_{280}. After the u-PA–coupled gels had been washed extensively, labeled or unlabeled extracted proteins were incubated with the gel for 16 hours at 4°C. Control experiments were performed in parallel using uncoupled Sepharose to determine the amount of nonspecific trapping by the Sepharose beads. When radiolabeled proteins were analyzed, the gel was washed extensively until the eluted radioactivity reached a stable background. Residual proteins bound to the u-PA–Sepharose gel or control Sepharose gel were eluted with either 50 mmol/L glycine buffer (pH 3.0) or 50 mmol/L citrate buffer (pH 3.0) in 0.1 mol/L NaCl and neutralized immediately with one-fifth vol HEPES buffer (pH 9). The eluted proteins were concentrated using Amicon YM-10 concentrating filters (W.R. Grace, Danvers, MA) before further analysis. In some experiments, u-PA binding proteins were first isolated using u-PA–Sepharose. The proteins were then labeled with Na\textsuperscript{125}I and iodogen beads and analyzed using SDS-PAGE and autoradiography. Gels used to analyze 	extsuperscript{125}I-labeled proteins were treated with Autofluor (National Diagnostics, Manville, NJ) before autoradiography. Replicate aliquots of eluted unlabeled proteins were separated using SDS-PAGE, stained with Coomassie brilliant blue (BioRad), and scanned using a laser densitometer (LKB Biotechnology, Gaithersburg, MD).

**Western Blotting Experiments**

To determine the immunologic relationship between the u-PA receptor on endothelial cells with the u-PA receptor expressed on PMA-stimulated U-937 cells, we performed Western blotting experiments with preimmune IgG and a polyclonal IgG anti-u-PA receptor antibody (both purified by protein A-Sepharose chromatography) raised in a rabbit to purified u-PA receptor protein isolated from PMA-stimulated U-937 cells.25 Extracts of PMA-stimulated U-937 cells or HUVEC in CHAPS were first separated using 10% SDS-PAGE (200 μg protein per lane), transferred to nitrocellulose, and incubated with the primary antibody (20 μg/mL) at 4°C. After washing, the antibody was identified using a commercial detection kit (Amersham, Arlington Heights, IL). To determine if our purified protein had significant contamination with u-PA or with PAI, we also tested whether isolated u-PA receptor protein reacted with monoclonal antibodies (MoAbs) to u-PA (a gift from M. L. Noll, Lepetit Research Center, Genzano, Italy), polyclonal antibodies to PAI types 1 and 2 (PAI-1, PAI-2, a gift from T. C. Wun, Monsanto, St. Louis, MO), polyclonal antibody to protase nexin 1 (a gift from D. Knauer, University of California at Irvine), or normal rabbit IgG by Western blotting.24 Prelabelled molecular mass standards (BioRad) were run on the gels in parallel and were readily visible after transfer to nitrocellulose.
Dot Blot Assay for u-PA Receptor Activity

Crude endothelial cell extract, purified u-PA receptor protein, BSA, or PBS alone was blotted onto nitrocellulose paper that had been premoistened with PBS. The nitrocellulose paper was washed twice with PBS and incubated with PBS containing 1% powdered milk (Carnation) and 0.1% Tween 20 for 30 minutes at 22°C. The nitrocellulose was then incubated with 125I-scu-PA (2 to 5 nM/mL, specific activity 2.0 × 10^6 cpm/μg) for 2 hours at 22°C, washed four times, and analyzed using autoradiography. In parallel experiments, portions of the nitrocellulose were incubated with 125I-scu-PA and a 50-fold molar excess of either scu-PA or rt-PA, or with 125I-rt-PA21 (11 nmol/L, specific activity 0.6 × 10^6 cpm/μg) with or without 50 molar excess unlabeled rt-PA.

Determination of the Apparent Molecular Mass of Active Solubilized Receptor Protein

To estimate the molecular mass of the solubilized u-PA receptor under non-denaturing conditions, endothelial cell proteins were extracted in CHAPS as described above and centrifuged at 100,000 g for 1 hour. The supernatant was applied to a 2.5 × 100-cm S-200 HR column (LKB Biotechnology) preequilibrated with PBS containing 0.01% CHAPS. The fractions were collected, and the total protein concentration was determined using the BCA protein assay (Pierce). The same fractions were assayed for receptor activity using the dot blot assay described above. The relative amount of activity determined was determined by scanning the autoradiogram with a laser densitometer (LKB Biotechnology).

Synthesis of u-PA Receptor-Specific cDNA

We used the polymerase chain reaction (PCR) to amplify full-length and partial cDNA transcripts encoding the u-PA receptor. Total cellular RNA (5 μg) was isolated from HUVEC and from U-937 cells incubated with PMA (100 nmol/L) for 48 hours by chloroform phenol extraction.30 cDNA was prepared using an oligo-dT primer (5' TTTTTTTTATTTTTTTTTT'3') containing a HindIII restriction site (this and subsequent restriction sites shown in bold type), and Moloney murine leukemia virus RNase H-reverse transcriptase (BRL, Gaithersburg, MD) following the manufacturer's suggestions. u-PA receptor-specific cDNA was amplified in a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT) using a DNA amplification kit (Perkin Elmer Cetus) and specific primers. The oligonucleotide sequence of the primers was based on the sequence of the u-PA receptor cDNA cloned from a fibroblast cDNA expression library.15 u-PA receptor-specific cDNA was amplified in a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT) using a DNA amplification kit (Perkin Elmer Cetus) and specific primers. The oligonucleotide sequence of the primers was based on the sequence of the u-PA receptor cDNA cloned from a fibroblast cDNA expression library.15 To prepare full-length cDNA, we used a 5' primer (5'GTGCCCAACCTCATGGGTCACCGY) and a 3' primer (5'GTGGTGTTGCACAGCAGAGGGG3') beginning at nucleotide 34, to which a HindIII restriction site was added (coding region begins at nucleotide 47) and a 3' primer corresponding to nucleotides 1,064 through 1,087 (5'GAATCCAGATCAGCAGAGAGGGG3'; stop codon is at position 1052 through 1054). We also amplified a smaller cDNA sequence for Southern and Northern blot analyses. The 5' primer (5'GTGGTGTTGCAGCAGAGAGGAGG3') of the smaller probe corresponded to nucleotides 485 through 499, whereas the 3' primer (5'GTATCCAGTTCCTGCGAGAGGGG3') corresponded to nucleotides 1,064 through 1,087 to which a HindIII restriction site was added. cDNA prepared from HUVEC and PMA–U-937 cells was subjected to 30 cycles of amplification in a DNA thermal cycler using a step program (94°C 1 min, 52°C 1 min, 72°C 1 min) with a final extension for 8 minutes at 72°C. The PCR products were analyzed by agarose gel electrophoresis, restriction mapping, and Southern and Northern blotting. According to the published sequence,14 unique restriction sites within the PCR product would be predicted for BamHI, AvaI, and PvuII (BRL) at positions 498 to 499, 571-572, and 699-700 respectively, which, on digestion of the full-length (1,054 bp) product should yield fragments of 538 and 516 bp for AvaI, 668 and 388 for PvuII, and 589 and 465 for BamH1. Digestions were performed for 1 hour at 37°C. The products were analyzed by electrophoresis on a preparative agarose gel containing ethidium bromide and were visualized by UV fluorescence. For Southern blot analysis, an internal, end-labeled oligonucleotide was used as a probe, which was homologous to nucleotides 61 through 634 (5'GGTTGTTGGTGCAGATTTGCAG3'). For Northern blot analysis, the 603-bp PCR product amplified from PMA-stimulated U-937 cell RNA (homologous to nucleotides 485 through 1,087) was gel purified and labeled by nick translation (BRL). HaeIII fragments of dsx174 DNA (BRL) were run in parallel for size comparisons.

Northern Blot Analysis

Total cytoplasmic RNA was isolated from HUVEC and from PMA-stimulated U-937 cells and resolved using agarose/formaldehyde gel electrophoresis.30 The RNA (20 μg) was then transferred to GeneScreen Plus (DuPont, Boston, MA) prehybridized in 20 x SSC, 50% formamide, and 5 x Denhardt’s solution containing 250 μg/mL sonicated Salmon sperm DNA (Sigma) at 42°C for 2 hours.31 The 603-bp PCR product, amplified from PMA-stimulated U-937 cells, was labeled by nick translation. The filter was hybridized overnight with the labeled probe at 42°C. The filter was then washed three times with 2 x SSC and 0.1% SDS at room temperature for 15 minutes, then washed three times with 0.1 x SSC 0.1% SDS at 50°C for 15 minutes, and then exposed to Kodak XAR film at -70°C for 3 days. The relative amount of u-PA receptor mRNA present was determined by densitometric analysis of the autoradiogram as compared with the total amount of RNA present prior to transfer.

Studies on u-PA Receptor Glycosylation

To determine if the u-PA receptor protein was glycosylated, we performed the following two experiments. First, HUVEC were passaged and grown for 48 hours in methionine-deficient media supplemented with 35S-methionine with and without 1 μg/ml tunicamycin (Calbiochem, San Diego, CA) to inhibit glycosylation. The labeled HUVEC u-PA receptor protein was then isolated by affinity chromatography as described above and analyzed by SDS-PAGE and autoradiography.

Second, the purified, 125I surface-labeled u-PA receptor protein or control glycoproteins (rt-PA and asialofetuin) were treated with various glycosidases and then analyzed using SDS-PAGE and autoradiography. Ten-microliter samples of surface-labeled receptor or control proteins in a total volume of 22 μL were incubated with 20 mU neuraminidase (from Clostridium perfringens, type X, Sigma) at 37°C with or without 1.0 mU O-glycanase (endo-W-N-acetylgalactosaminidase, Boehringer Mannheim, Indianapolis, IN) in cacosylate buffer (20 mmol/L, pH 6)32 or with 0.5 mU N-glycanase (peptide-N-glycosidase F, Genzyme, Boston, MA)33 in phosphate buffer (0.25 mol/L, pH 8.6) containing NP-40 (1.4%) and α-mannosidase (10 mmol/L). Digestions were conducted for 46 hours with half of the enzyme added at the beginning of the incubation and the remainder added after 24 hours. All digestions were conducted in the presence of 2 mol/L PMSF. Samples of treated and untreated proteins were then analyzed using SDS-PAGE and autoradiography.

RESULTS

Estimation of Molecular Mass of u-PA Receptor Using Chemical Cross-linking

Endothelial cells express binding sites to which u-PA binds saturably and reversibly through a region within the amino-
terminal portion of the molecule that appears to require the epidermal growth factor-like domain. The catalytic site has been chemically blocked. U-PA also binds to PAI-I expressed on the surface of HUVEC, however, forming 1:1 complexes that are stable in SDS. Therefore, to identify the u-PA receptor, we used scu-PA (in which the catalytic site is not available), DIP-tcu-PA (in which the catalytic site has been chemically blocked), and the ATF fragment of U-PA (which contains only the first 143 amino acids of U-PA and therefore lacks the catalytic site) as ligands that compete with each other for binding to HUVEC and that do not bind to PAIs.

To obtain an estimate of the molecular mass of the u-PA binding protein expressed on the cell surface, 125I-DIP-tcu-PA and 125I-ATF were incubated with monolayers of HUVEC under equilibrium binding conditions and were cross-linked to the cell surface. In the absence of a cross-linking reagent, 125I-DIP-tcu-PA migrated at its native molecular mass (Fig 1, lane 1), consistent with its reversible association with the cell surface as previously described. In the presence of the cross-linking reagent, however, an increase in the apparent molecular mass from 55 to approximately 95 Kd was noted (lane 2). An additional band was noted at the top of this 10% gel. When excess unlabeled tcu-PA was present, specific binding was inhibited (data not shown). When 125I-ATF was used as a ligand, the apparent molecular mass increased from 19 Kd (lane 3) to approximately 60 Kd (lane 4) after addition of the cross-linking reagent. No specific binding was observed when excess unlabeled ligand was included in the cross-linking experiment (lane 5). These results suggest that the u-PA receptor, or at least a ligand binding subunit of the receptor, migrates with an apparent molecular mass of approximately 40 Kd.

**Isolation of u-PA Binding Protein**

**Preparative isolation.** u-PA binding proteins were isolated from 3 to 8 \( \times 10^6 \) HUVEC using affinity columns to which 10 mg purified scu-PA had been linked. After extensive washing, the u-PA binding proteins were then eluted using a brief exposure to pH 3 which has previously been demonstrated to reverse the binding of u-PA to its receptor on U-937 cells. A portion of the eluted protein was radiolabeled with 125I and analyzed using SDS-PAGE and autoradiography. A labeled protein having an apparent molecular mass of approximately 36 Kd was the major band detected on autoradiography (Fig 2, inset), and a small amount of radioactivity was detected at the solvent front. The purified proteins were also analyzed after SDS-PAGE by staining with Coomassie blue. The band at approximately 36 Kd appeared to be composed of three proteins having molecular masses of 37, 35.5, and 34.5 Kd that contained similar amounts of stainable protein determined by densitometry (Fig 2). In five such preparations, the presence of a dominant band at approximately 36 Kd was a consistent finding. Additional bands of variable intensity migrating with apparent molecular masses of approximately 80 Kd, 46 Kd, and 32 Kd were also noted on longer autoradiographic exposures (not shown).

**Surface labeling.** To detect whether each of these proteins was expressed on the external surface of endothelial cells, we labeled intact monolayers of HUVEC with 125I and isolated the receptor protein using scu-PA-Sepharose affinity chromatography. When the surface-labeled proteins were analyzed using SDS-PAGE and autoradiography, only two bands having molecular masses of 46 and 180 Kd were detected (Fig 3). No protein having a molecular mass in the

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**Fig 1.** Crosslinking of u-PA to endothelial cells. HUVEC were incubated for 2 hours at 4°C with DIP-tcu-PA or ATF that had been radiolabeled (+) with NaI. After washing, the cells were incubated in PBS buffer with or without 1 mmol/L disuccinimidyl suberate (cross-linking agent) at 22°C for 15 minutes and then with 10 mmol/L ammonium acetate for 10 minutes. Binding of labeled ATF in the presence of 50-fold molar excess unlabeled (cold) ATF is shown in lane 5. The cells were solubilized in 0.5% Triton X-100 and analyzed using 10% SDS-PAGE under reducing conditions followed by autoradiography. The molecular mass (Kd) of each protein is shown (left) and was estimated from standards run in parallel.
Fig 2. \(^{125}\text{I}-\)labeled purified u-PA receptor protein. Unlabeled HUVEC (8 x 10^6) were solubilized in 0.5% CHAPS detergent in the presence of protease inhibitors. Receptor protein was isolated using an affinity column prepared with 10 mg purified scu-PA immobilized on Sepharose. The u-PA binding fraction was concentrated, and a 50-\(\mu\)l fraction was radiolabeled with Na\(^{125}\text{I}\) and separated from unbound iodide by a PD-10 column. The labeled proteins were then separated using 10% SDS-PAGE under reducing conditions and analyzed using autoradiography (inset). A sample of the unlabeled isolated receptor protein was also separated using 10% SDS-PAGE under reducing conditions and stained with Coomassie blue. The gel was then scanned with a laser densitometer. Three closely associated peaks of approximately equal amplitude were identified, with apparent molecular masses of 37, 35.5, and 34.5 Kd estimated from molecular mass standards run in parallel.

36-Kd range was detected. Radioactivity migrating at the solvent front (<14 Kd) was also present in some gels; it was not dialyzable using a membrane with a pore exclusion size of 10 Kd. This band was also noted when metabolically labeled proteins were isolated using similar techniques.

Receptor activity. Intact endothelial cells bind both radiolabeled t-PA\(^{21,24,32}\) and u-PA.\(^{12,13}\) We and other investigators previously reported that t-PA does not inhibit u-PA binding to certain cells\(^{12,13}\) whereas u-PA does inhibit binding of t-PA.\(^{21,32}\) Thus, we expect that a u-PA affinity column might copurify both u-PA and t-PA binding proteins. To address this possibility, proteins extracted from HUVEC and the eluate from the scu-PA-Sepharose column were blotted onto nitrocellulose paper and incubated with either radiolabeled scu-PA or rt-PA. In control experiments, \(^{125}\text{I}-\)scu-PA did not bind specifically either to unlabeled BSA or to buffer blotted onto nitrocellulose. However, \(^{125}\text{I}-\)scu-PA bound specifically in a dose-dependent fashion both to the extracted cellular proteins and isolated u-PA binding proteins eluted from the affinity column (Fig 4, column 1). Fifty molar excess unlabeled scu-PA inhibited binding of labeled scu-PA to both preparations (column 2), whereas excess unlabeled rt-PA did not (column 3). Radiolabeled rt-PA did not bind to the extract or to the eluted protein with or without addition of unlabeled rt-PA under the same experimental conditions (columns 4 and 5). This suggests that the predominant proteins isolated were u-PA specific binding proteins, although a protein to which t-PA also binds weakly may have been copurified.

Molecular mass determination of intact solubilized receptor protein. To determine the apparent molecular mass of the u-PA binding protein under less denaturing conditions,
solubilized endothelial cell proteins were separated using S-200 HR chromatography. The scu-PA binding activity in the individual fractions was analyzed using the dot blot assay described above, and the relative activity was estimated with laser densitometry. Activity was found in two protein peaks (Fig 5). The highest specific activity (u-PA binding activity per microgram of protein) was found in fractions migrating with an apparent molecular mass between approximately 36 and 46 Kd; these fractions comprised about 50% of the total receptor activity. The remaining activity was detected in the void volume (see below).

**Interrelationship Among u-PA Binding Proteins**

The previous experiments demonstrate that u-PA receptors with several different molecular masses were isolated from HUVEC under various conditions. The predominant protein isolated from whole cell lysates migrated with an apparent molecular mass of approximately 36 Kd, whereas the predominant species expressed on the cell surface migrated at approximately 46 Kd. These differences could indicate that endothelial cells express more than one distinct u-PA binding protein or that differences in glycosylation or other posttranslational modifications of a single protein could be responsible for at least a portion of the observed heterogeneity. Therefore, we performed a series of experiments to distinguish between these two possibilities.

**Immunologic identification.** We first investigated whether the various protein bands isolated by ligand affinity chromatography from HUVEC were similar antigenically to each other and to the u-PA receptor previously isolated from...
HUMAN ENDOTHELIAL CELL u-PA RECEPTOR

1 2 3 4

-130 -75 -50 -39 -27 -17

Fig 6. Western blotting analysis. Whole cell detergent extracts (200 μg total protein per lane) of PMA-stimulated U-937 cells (lanes 1 and 2) or HUVEC (lane 3 and 4) were separated on a single 10% SDS polyacrylamide gel and transferred to nitrocellulose. The lanes containing cell extract were overlaid with either preimmune IgG (lanes 1 and 3, 20 μg/mL) or anti-u-PA receptor IgG (lanes 2 and 4, 20 μg/mL). Antibody was detected using a commercial enzyme linked secondary antibody detection system (Amersham). The position of the molecular mass standards are shown (right).

Fig 7. Restriction digest of PCR amplified full-length u-PA receptor cDNA. cDNA was prepared from HUVEC and PMA-stimulated U-937 cell RNA by reverse transcription. u-PA receptor cDNAs were amplified by the PCR using oligonucleotide primers, as described in the Materials and Methods section, to generate u-PA receptor cDNAs corresponding to nucleotide positions 34 through 1,087 of the published sequence of a cloned human fibroblast u-PA receptor cDNA.16 Lanes 2 and 7 demonstrate the PCR reaction product for HUVEC and PMA-stimulated U-937 cells, respectively. The published sequence also predicts that single restriction sites exist for the three endonucleases Aval, PvuII, and BamHI within this region. The PCR products were digested with Aval (lanes 3 and 8), PvuII (lanes 4 and 9), and BamHI (lanes 5 and 10), separated by agarose gel electrophoresis containing ethidium bromide, and visualized with UV fluorescence. Molecular mass markers (HaeIII-digested φX174 RF DNA) are shown in lanes 1, 6, and 11.

PMA-stimulated U-937 cells. Whole cell extracts from both cell types were analyzed in parallel by Western blotting using a polyclonal anti-u-PA receptor antibody.29 A prominent band at approximately 47 Kd was labeled in both the PMA-U-937 cell (Fig 6, lane 2) and the endothelial cell preparations (lane 4). A broader dark band of protein in the 55- to 70-Kd region was evident in both lanes as well. Bands at about 37 and 32 Kd were also noted in the receptor preparation from HUVEC and corresponding bands were also identified in this region in the PMA-U-937 cell preparation. None of these bands were labeled by preimmune serum or secondary antibody alone. Some staining was noted at the solvent front and at 20 Kd but was also noted with preimmune serum. Antibodies to PAI-1, PAI-2, protease nexin-1, or u-PA failed to react to purified receptor preparations by Western blotting (not shown). These results suggest that each of the u-PA binding proteins identified on or in HUVEC are derived from a single protein that is antigenically similar to the receptor isolated from PMA-stimulated U-937 cells.

Estimate of u-PA receptor size by the PCR. To estimate the expected size of unglycosylated u-PA receptor protein, we amplified a full-length receptor-specific cDNA from HUVEC and PMA-stimulated U-937 cells using the PCR and oligonucleotide primers homologous to nucleotide positions 34 through 48 and 1,064 through 1,087 from the u-PA receptor cDNA cloned from a transformed human fibroblast cDNA expression library.16 A single PCR product of approximately the expected size (1,054 bp) was isolated from both cell types (Fig 7, lanes 2 and 7). Homology between these two PCR products and the previously cloned fibroblast cDNA sequence was confirmed by Southern blot analysis using an end-labeled, internal oligonucleotide (not shown) and by restriction enzyme mapping using Avai (Fig 7, lanes 3 and 8), PvuII (lanes 4 and 9), and BamHI (lanes 5 and 10), each
of which is predicted to have a single restriction site within the sequence.\textsuperscript{16} As shown in Fig 7, restriction digestion of both PCR products produced fragments migrating with apparent sizes closely approximating the predicted size (538 and 516 bp for \textit{Ava}I, 666 and 388 for \textit{Pst}II, and 589 and 469 for \textit{Bam}H1). The predicted molecular mass of an unglycosylated protein encoded by a cDNA of this size on average would be approximately 38.5 Kd.

\textit{Northern blot analysis.} The predicted size of the unmodified protein was also consistent with the results of the Northern blot analysis. Hybridization of total RNA isolated from endothelial cells and PMA-stimulated U-937 cells with a radiolabeled 603-bp PCR product identified a single, mRNA transcript of approximately 1.4 kb from both cell types (Fig 8). Although before transfer UV fluorescence confirmed the presence of equal amounts of RNA loaded per lane, the PMA-stimulated U-937 cells expressed approximately 20 times as much u-PA receptor mRNA measured by densitometry as did endothelial cells.

\textit{Metabolic labeling: Effect of tunicamycin.} The data suggest that the higher molecular mass of the u-PA receptor isolated from the cell surface compared with that isolated from whole cell lysates may be the result of posttranslational modifications such as glycosylation. To investigate this possibility, proteins from endothelial cells grown with or without tunicamycin (1 \( \mu \)g/mL) were metabolically labeled with \textsuperscript{35}S-methionine and solubilized in CHAPS. The u-PA binding proteins were then isolated with scu-PA–Sepharose and analyzed with SDS-PAGE and autoradiography. When proteins from control HUVEC were analyzed, a broad band at about 46 Kd was detected in addition to the predominant band at about 36 Kd and minor bands at about 32 and 80 Kd (Fig 9). When HUVEC were grown in media containing tunicamycin, the predominant u-PA binding protein continued to migrate with a molecular mass of about 36 Kd; however, two changes were noted. First, the 46-Kd band was no longer detected. Second, there was an increase in the relative amount of the higher mol-wt species detected, although this protein now migrated with a somewhat lower apparent molecular mass (~75 Kd). The intensity of the other minor bands was essentially unchanged. These data are consistent with the hypothesis that the 46-Kd species results from glycosylation of a u-PA binding protein having a lower molecular mass. The potential origin of the higher molecular mass forms is discussed below.

\textit{Endoglycosidase experiments.} To test this hypothesis further, we incubated \textsuperscript{125}I surface-labeled u-PA binding proteins with various glycosidases. (This labeled u-PA receptor preparation was the same as that shown in Fig 3). The molecular mass of the purified protein did not change substantially after incubation with neuraminidase (Fig 10, lane 2) alone or in combination with O-glycanase (Fig 10, lane 3), although asialofetuin incubated under the same conditions migrated with a reduced molecular mass as expected (not shown). However, when the u-PA binding proteins were incubated with N-glycanase in the presence of protease inhibitors, an additional band of lower molecular mass (<40 Kd) was readily detected (Fig 10, lane 4).

\section*{Discussion}

Receptors for u-PA are expressed by many cell types, such as endothelial cells. These receptors permit u-PA to bind to cell surfaces with high affinity.\textsuperscript{12,13,36} Cell-bound scu-PA converted to two-chain u-PA by plasmin appears to retain enzymatic activity, even though PAIs can be detected in the extracellular environment.\textsuperscript{4} The identity of the u-PA receptor expressed by endothelial cells and its relationship to receptors on other cell types has not been determined.

Variation in both the affinity of scu-PA binding to different cell types and in the apparent molecular mass of u-PA receptors isolated from U-937 cells (~55 to 60 Kd), HeLa cells (~45 ± 5 Kd) and the receptor identified by ligand blotting in lysates of endothelial cells (~48 Kd) have been reported.\textsuperscript{13,17,18} The u-PA receptor associated with each of these cells appears to migrate with a higher apparent molecular mass than would be expected based on the cDNA sequence described recently.\textsuperscript{16} The observed heterogeneity may reflect the existence of more than one type of high-affinity receptor or differences in posttranslational processes such as glycosylation among the cell types studied. Thus, we sought to isolate and characterize the u-PA receptor expressed by human endothelial cells and to determine its relationship to the receptor expressed on PMA-stimulated U-937 cells that had been characterized previously.\textsuperscript{19}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure8.png}
\caption{ Autoradiograph of Northern blot. Total cellular RNA (20 \( \mu \)g) was isolated from HUVEC (lane 1) and from PMA-stimulated U-937 cells (lane 2) and resolved by agarose/formaldehyde gel electrophoresis as described in the Materials and Methods section. The blot was hybridized with an \textsuperscript{125}P-labeled 603-bp PCR fragment amplified from PMA-stimulated U-937 cells as described. The positions of the ribosomal RNA are shown (right).}
\end{figure}
HUMAN ENDOTHELIAL CELL u-PA RECEPTOR

Fig 9. Effect of tunicamycin on the molecular mass of the u-PA receptor protein. Endothelial cell proteins were metabolically labeled with [35S]-methionine for 48 hours with or without tunicamycin (1 μg/mL). The cells were solubilized, and u-PA binding proteins were isolated by scu-PA-Sepharose affinity chromatography. The proteins were analyzed after reduction with 2-mercaptoethanol using 10% SDS-PAGE, followed by enhancement with Autofluor and autoradiography. Labeled proteins isolated from the control HUVEC and HUVEC incubated with tunicamycin are shown in lanes 1 and 2, respectively. The 14C-labeled molecular mass standards are shown in lane 3.

Fig 10. Effect of glycosidases on the molecular mass of u-PA receptor protein. Surface-labeled u-PA binding proteins (same material as shown in Fig 3) were incubated with buffer (lane 1), neuraminidase (lane 2), neuraminidase and O-glycanase (lane 3), or N-glycanase (lane 4) for 46 hours at 37°C with protease inhibitors added as described. The proteins were then analyzed using 10% SDS-PAGE and autoradiography after reduction with 2-mercaptoethanol. Molecular mass standards run in parallel are shown (right).
Initially, we estimated the molecular mass of the u-PA receptor on endothelial cells to be about 40 Kd, based on the change in the apparent mobility of radiolabeled catalytic site-blocked tcu-PA or ATF cross-linked to the cell surface. A protein (or proteins) with a similar molecular mass was isolated from lysates of metabolically labeled or surface-labeled endothelial cell proteins by ligand affinity chromatography. The proteins that eluted from the ligand affinity column bound scu-PA but did not bind rt-PA at the concentrations tested using a dot blot ligand binding assay. The same specificity was observed when intact cells were studied.12,13

Small but consistent differences were detected in the apparent molecular mass of the u-PA receptor isolated from surface-labeled cells (≈46 Kd) and the molecular mass of the predominant u-PA binding protein isolated from whole cell lysates (≈36 Kd). Moreover, the binding protein of approximately 36 Kd was composed of at least three distinct protein bands migrating between 34 and 37 Kd when stained with Coomassie blue. At least two of these may represent u-PA receptor with and without cleavage of the signal peptide or mature u-PA receptor with none, one, or two of its potential glycosylation sites modified. Less prominent high molecular mass bands were also found at about 80 and 180 Kd when metabolically labeled and surface-labeled proteins were analyzed, respectively.

The data obtained from the S-200 chromatography column provides additional support to the notion that the solubilized u-PA receptor, or a ligand binding subunit of the receptor, migrates with an apparent molecular mass of about 36 to 46 Kd. The peak of activity observed in the void volume may represent receptor in incompletely solubilized membrane fragments or in detergent micelles; however, the intact receptor may also be a much larger structure with noncovalent attachments to other subunits or to other proteins. Another possibility is that the receptor can self-associate and form multimers.

Based on these data, we hypothesized that either each of these u-PA binding proteins was unrelated or that the endothelial cell u-PA receptor is synthesized as a protein of about 36 Kd and is modified posttranslationally before or subsequent to its expression on the luminal surface as a glycoprotein of about 46 Kd. We then performed a series of experiments to distinguish between these possibilities.

First, we observed that a polyclonal antibody against a u-PA receptor isolated from PMA-stimulated U-937 cells19,20 recognized both the approximately 36- and 46-Kd proteins as well as a broad protein band of somewhat higher molecular mass on Western blot analysis. The same pattern of binding was observed when lysates from HUVEC and PMA-stimulated U-937 cells were analyzed in parallel. Second, the cDNA amplified from both HUVEC and U-937 cells by PCR yielded a single product of identical size. The identity of this PCR product as u-PA receptor cDNA was demonstrated by Southern blot hybridization with an internal oligonucleotide probe generated from the published sequence18 as well as by generation of restriction fragments of the expected size specific for the u-PA receptor cDNA target sequence.18 These results suggest that a high degree of homology exists between the u-PA receptors in these two cell types and that reported for the fibroblast u-PA receptor. Third, a single transcript of about 1.4 kb was identified by Northern blot analysis of RNA isolated from both cell types, again consistent with published analyses of PMA-stimulated U-937 cells.18

These studies suggest that at least some of the heterogeneity that we observed in the u-PA binding proteins after ligand affinity chromatography and on Western blot analysis are more likely to result from variation in posttranslational modification, such as the extent of glycosylation, than they are to reflect products of different genes, although these studies do not exclude the possible existence of other distinct u-PA binding proteins. In support of the notion that glycosylation was at least partially responsible for the observed heterogeneity, we found that the 46-Kd band was no longer detected when endothelial cells were grown with tunicamycin added during the metabolic labeling, whereas the intensity of the 36-Kd band was unaffected. Moreover, the molecular mass of the surface-labeled receptor was reduced to less than 40 Kd after the isolated receptor protein was incubated with N-glycanase.

These observations are consistent with the results of two recent studies. First, the molecular mass of the unglycosylated receptor expressed by transformed fibroblasts16 is predicted to be 37 Kd, or 34.6 Kd after removal of the putative signal peptide based on the experimentally determined N-terminal amino acid sequence.19 Thus, if the precursor had u-PA binding activity as well, this might explain some of the microheterogeneity found in molecular mass in the range of 32 to 37 Kd. Second, the deduced amino acid sequence also indicates the presence of five potential N-glycosylation sites. The apparent molecular mass of the u-PA receptor isolated from U-937 cells was reduced from 55 to 60 Kd to about 35 Kd under severe reducing and denaturing conditions which are required to deglycosylate the molecule fully, presumably because of a high degree of internal disulfide bonding.19 Different degrees of glycosylation could also explain some of the heterogeneity we found.

Complex glycosylation does not appear to be required for scu-PA to bind to the receptor because the 36-Kd form, which was expressed by cells grown in tunicamycin, was readily isolated by ligand affinity chromatography. These studies do not exclude the possibility that part of the heterogeneity in the affinity of u-PA for its receptors on various cell types may be the result of specific changes in the pattern or extent of glycosylation or other posttranslational modifications. Moreover, we have not excluded the possibility that microheterogeneity exists somewhere within the sequence of the various binding proteins, which could have important consequences for ligand affinity.

The identities of the bands of about 80 and 180 Kd, the band of less than 14 Kd, as well as the high-molecular-mass cross-linked band are unclear. Based on apparent molecular mass, the 80- and 180-Kd proteins may represent homodimers and homotetramers of the receptor or of the ligand binding portion of the receptor that form intracellularly or...
form in vitro during receptor isolation. The observation that these higher molecular mass species were most evident after the protein had been incubated with N-glycanase (Fig 5) or after HUVEC had been incubated with tunicamycin (Fig 6) suggests that deglycosylation might enhance the rate of polymerization or make the polymers less susceptible to reduction by 2-mercaptoethanol. Broad bands in the 55- to 70-Kd region by Western blot analysis were noted for both HUVEC and PMA-stimulated U-937 cells. The u-PA binding proteins which migrate in this region may result from more extensive or alternate patterns of glycosylation. These higher molecular mass proteins may also be unrelated to the predominant 36-Kd u-PA receptor and represent other u-PA binding proteins or even t-PA binding proteins, because u-PA competes for t-PA binding to endothelial cells. The failure of t-PA to bind to the receptor proteins on dot blot ligand binding assay makes this less likely, although it could reflect the lower affinity of t-PA for its receptor or other changes in the protein which occurred during its isolation. The significance of the band at approximately 10 to 13 Kd is also uncertain. It may represent a proteolytic fragment of the 46-Kd receptor that migrates near the solvent front or it may represent a distinct u-PA binding protein. The definitive answer will require that the amino acid sequence of each of these proteins be determined individually.

Receptors for plasminogen activators may help localize PA activity on cell surfaces by protecting the proenzyme from rapid inactivation by pericellular and/or extracellular plasminogen activator inhibitors or by linking the protein to intracellular events; eg, the observation that expression of u-PA appears to be greatest at focal contact points and at the leading edge of migrating cells may indicate that u-PA receptors can associate with elements of the cytoskeleton under some conditions or migrate in a directional fashion within the membrane in response to specific agonists. The observation that the susceptibility of u-PA to inhibition by certain PAIS may be altered when receptor bound is still controversial and may involve both partitioning of the enzyme and inhibitor within the membrane as well as modifications in the u-PA molecule that affect the availability of the active site to inhibitor and substrate. Endothelial cells may also facilitate the conversion of scu-PA to tcu-PA by localizing both scu-PA and plasmin(ogen) near the cell surface through receptor-mediated interactions. The precise role that endothelial cell u-PA receptors play in each of these processes will require isolation of sufficient protein to allow direct performance of such experiments.

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